Title: Applications with and methods for producing
selected interstrand cross-links in nucleic acids

Field of the invention

The invention lies in the field of nucleic acid cross-
linking and uses thereof. More specifically the invention
relates to method for producing selected interstrand cross-
links in nucleic acids and uses thereof. One important aspect
of the invention relates to the use of selected interstrand
cross-links for the selective amplification of certain nucleic
acids in an amplification reaction.

Background of the invention

Many different compounds have been identified that posses
nucleic acid cross-linking activity. Cross-linking of nucleic
acids is most commonly used for therapeutic purposes in the
intervention with proliferative disorders such as cancer. Most
cross-linking agents cross-link nucleic acids in very specific
ways and on specific places in nucleic acids. However, the
frequency of these specific places in most nucleic acids are so
high that effectively the cross-links are provided throughout
the nucleic acid molecules. For the use of these cross-linking
compounds in the intervention of cancer this so-called
apparently random cross-linking activity does not prevent some
kind of a therapeutic effect. However, in the ideal situation
cross-links would only be applied in the nucleic acid of the
cells of which the proliferation should be interfered with. For
instance by applying the cross-links only to those nucleic
acids involved in the transformation of said cell, i.e. the
oncogenes or the RNA of said oncogenes. Such specificity was

not possible with the current methods of cross-linking. The apparent random cross-linking activity of cross-linking agents also prevents the use of these compounds in assays that require more specific cross-linking. In one aspect the invention

5 provides a method for producing cross-links in selected regions of a nucleic acid. In one aspect said method may be used to prevent at least in part, certain regions in a nucleic acid from taking part in a process such as, but not limited to a process comprising a hybridisation or an amplification or both.

10 In one aspect said method of producing selected interstrand cross-links is used in a process for producing a probe deprived at least in part of repetitive sequences. Such a probe is useful for the detection of for example nucleic acid sequences in chromosome painting in the field of cytogenetics.

15 The introduction of fluorescence *in situ* hybridisation (FISH) has significantly changed cytogenetics. Human FISH karyotyping is now successfully applied to elucidate complex chromosome rearrangements. Multi-colour FISH analysis of chromosomes is not necessarily restricted to the use of whole

20 chromosome paints. Recently, sets of probes have been generated that specifically recognise the (sub)telomeric regions of a particular chromosome and that are applied in a multi-colour FISH format to detect cryptic translocations, frequently occurring in mental retardations.

25 The selective staining of 24 human chromosomes is at present accomplished through binary combinations of probes that are labelled with 5 distinct fluorophores (Schroeck *et al.*, 1996; Speicher *et al.*, 1996).

For this so-called combinatorial labelling [also called

30 multiplex FISH] the number of recognisable targets (n) using (k) different fluorophores is n= 2^k-1 colours. Five fluorophores thus allow a maximum of 31 colours, sufficient to recognise 24 chromosomes, but insufficient for instance to

explore the use of p and q arm specific probes for the
detection of intrachromosomal rearrangements.

Thus, multi-colour FISH analysis of chromosomes would benefit
directly from a method to increase the number of simultaneously
5   recognisable targets beyond the 27 reported so far (Nederlof *et
al.*, 1992; Dauwerse *et al.*, 1992; Morrison and Legator, 1997).
Higher FISH multiplicity is achievable by ratio labelling. This
technique, by which a given probe is composed of a mixture of
probes with different fluorescent labels, has great potential.
10   As an illustration, one may consider the number of recognisable
colours that could be composed with the three primary colours
blue, green and red. In practice though, ratio labelling is
considerably more complex than combinatorial labelling.
Recognition of chromosomes stained with ratio labelled probes
15   is not a " yes or no colour" decision (as in the binary
approach) but requires accurate measurement of colour.

Summary of the invention

20   The present invention provides methods for the selected cross-
linking of nucleic acids. Specific regions in nucleic acids can
be selected and specifically cross-linked with minor or not
detectable "a-specific" cross-linking in not selected regions.
The method is used in one non-limiting application, for the
25   selected amplification of certain sequences from a pool of
potentially amplifiable sequences. The method is used in
another non-limiting application for the preparation of a probe
for the detection of nucleic acids wherein selected sequences
are at least in part prevented from taking part in a
30   hybridisation reaction. In one aspect the invention provides a
method for the generation of a probe for the detection of
chromosomes or parts thereof. In one non-limiting example of

such a probe said probe is labelled by means of one aspect of the COBRA technique of the invention.

Detailed description of the invention

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In one embodiment, the invention provides a process for producing selected interstrand cross-links in nucleic acids comprising hybridising single strand nucleic acid(s) with complementary single strand nucleic acid(s) or a functional

10 analogue thereof, wherein said nucleic acid(s) or said complementary nucleic acid(s) or both comprise a cross-linking agent. In a preferred embodiment of the invention only said single stranded nucleic acid(s) or said complementary nucleic acid(s) comprises a cross-linking agent. Said nucleic acid,

15 preferably said complementary nucleic acid, may also be a functional analogue of a nucleic acid. One such analogue comprises peptide nucleic acid (PNA). In a preferred embodiment of the invention said nucleic acid or said complementary nucleic acid or both are DNA. A preferred principle for

20 selecting a nucleic acid region is through hybridisation with one or more nucleic acids complementary to said region. Cross-links may be provided through a cross-linking agent. Cross-links may be provided by hybridising one or more complementary nucleic acids to a nucleic acid thereby selecting regions for

25 cross-linking, and contacting said nucleic acid with a cross-linking agent. The selected double stranded regions are cross-linked whereas the non-selected single stranded regions are not cross-linked. For some purposes excess cross-linking agent and/or cross-linking agent (or reaction intermediates) not

30 contributing to double stranded intermediates can be removed or inactivated before use of the selectively cross-linked nucleic acid.

Preferably, cross-linking agents are used that cross-link double stranded nucleic acids and that have minor or undetectable cross-linking activity in single stranded nucleic acids. Alternatively, cross-linking agent is linked to the one

5   or more complementary nucleic acids before hybridisation whereupon cross-linking of selected regions is achieved after hybridisation of said complementary nucleic acid to the selected region. Preferably, cross-linking activity of the cross-linking agent is low when the nucleic acid is a single

10   strand form and high when the nucleic acid is in a double stranded form.

Regions in a nucleic acid may be selected for cross-linking by adding complementary nucleic acid to the single stranded form of said nucleic acid and performing a hybridisation. However,

15   advantage may be taken of complementary regions in a nucleic acid in that said complementary regions are induced to hybridise to each other after said nucleic acid has been made single strand and allowed to hybridise. Particularly in this case, but not limited to this case, selection of different

20   regions can be varied by varying the hybridisation conditions. Regions may also be selected for cross-linking by using a combination of the methods mentioned above. For instance some regions may be selected through the addition of complementary nucleic acid(s), whereas other and or the same regions may be

25   selected through hybridisation of complementary regions within a nucleic acid.

It is of course essential that complementary nucleic acids comprises sequences that are complementary to a selected region in a nucleic acid. However, it is not necessary that all

30   sequences in the complementary nucleic acids are in fact complementary to a selected region in a nucleic acid. Extra sequences in said complementary nucleic acids may be useful for a specific purpose or may just be present for convenience as

long as they do not prevent the primary function of said complementary nucleic acids, i.e. hybridising to a selected region.

The level of cross-linking and the nature of the cross-link

5   determine tightness of the cross-linking between the selected nucleic acid region and the complementary nucleic acid. The required tightness of the cross-linking varies with the specific application of the invention. In applications wherein selected cross-linking is performed to prevent denaturation of

10   a selectively cross-linked double stranded nucleic acid, the tightness of the linking should be sufficiently high to at least in part prevent denaturation in conditions that would enable denaturation of the selected region without cross-linking.

15   In one embodiment of the invention the cross-linking agent comprises a transition metal, preferably platinum, capable of cross-linking double stranded nucleic acids. In a preferred aspect of this embodiment the cross-linking agent comprises (trans)-dichlorodiam(m)ineplatinum.

20   Trans-dichlorodiammineplatinum (II) (trans-DDP) can form intrastrand cross-links between adjacent base residues in a DNA strand (Cohen et al., 1980). The intrastrand cross-links between two guanine (G) residues, or between a G and a cytosine (C) residue, or between a G and a cytosine (C) residue

25   separated by at least one residue, are the most favourable ones (Eastman et al.,1988; Pinto et al., 1985; Lepre et al, 1987). The 1,3-intrastrand cross-links between trans-DDP and two G residues separated by one intermediate base are stable within single-stranded oligonucleotides. As soon as the platinated

30   oligonucleotides hybridise to their complementary strands the 1,3-intrastrand cross-links rearranges to form an interstrand cross-link (Dalbies et al., 1994). This interstrand cross-linking effect of trans-DDP can be used in a strategy to

selectively cross-link certain nucleic acid sequences in a pool
of nucleic acid sequences.

In one application of the invention selected interstrand cross-
5   links are provided in oncogenes present in the DNA of cells
that are transformed or in the process of transforming. In this
application said interstrand cross-links are provided to hamper
at least in part replication and/or transcription of said
oncogenes. Said application may be useful in the treatment or
10   prevention of cancer.

In another application of the invention is provided a method
for the selected interstrand cross-linking of selected
sequences in a nucleic acid sequence comprising hybridising at
least one selected single strand sequence with a complementary
15   single strand nucleic acid wherein said selected sequence or
said complementary nucleic acid or both comprise a cross-
linking agent, wherein said cross-linking hampers further
hybridisation and/or replication of said selected sequence.

In another application of the invention repetitive sequences in
20   a nucleic acid are selectively cross-linked to block the
amplification of the selected region(s).

For chromosome painting, chromosome-specific DNA is used as a
probe. This probe is generally obtained by performing a PCR
based amplification, such as but not-limited to DOP-PCR, on DNA
25   of specific chromosome, which is isolated by flow sorting or
micro-sectioning. Chromosomal DNA contains a lot of repetitive
sequences, like telomeric DNA, centromeric repeats, LINEs,
SINEs and VNTRs. During DOP-PCR, these repetitive sequences
will also be amplified and in *in situ* hybridisation
30   experiments, they will create differential labelling of all
chromosomes. Normally this background labelling is prevented by
adding repetitive DNA to the hybridisation mixture, which
consists of a pool of repetitive sequences. However, the

technique of using repetitive DNA for this purpose is not ideal because the background is not reduced completely and also the signal of the probe is reduced.

In order to circumvent the necessity of blocking during
5   hybridisation it would be desirable to exclude the presence of repetitive sequences in the probe DNA. Methods to remove repetitive sequences by subtraction are known in the art (Craig et al., 1997). Subtraction methods are not preferred because they require additional manipulation of the probe and
10   subtraction is not easy to reproduce. The problem with the current methods of producing a probe with a satisfactory low level of repetitive sequences is that during the generation of the probe, repetitive sequences are also generated. Since repetitive sequences are also generated in the probe measures
15   have to be taken to eliminate their hybridisation or to remove them from the probe. These measures have as yet problems as mentioned above. With the methods of the invention we have designed a novel approach to produce a probe, wherein the probe is generated under conditions that prevent or lower the amount
20   of repetitive sequences generated. This procedure results in a probe that has a lower contamination with repetitive sequences which (for most purposes) can be used directly. However, if further removal of repetitive sequences is desired the probe generated through the methods of the invention also presents a
25   better substrate for the subsequent prevention of hybridisation of repetitive sequences strategies or the removal of repetitive sequences from the probe, since the probe already was less contaminated with repetitive sequences to begin with.

30   The following is a non-limiting example of an aspect of the invention wherein a strategy of designing a probe with a low amount of repetitive sequences is described. DOP-PCR is performed with degenerative primers on chromosomal DNA,

isolated by flow sorting or micro-sectioning. During the DOP-
PCR the repetitive sequences are being cross-linked by trans-
DDP labelled nucleotide sequences, complementary to these
sequences, wherein said nucleotide sequences are preferably one
5   or more trans-DDP labelled oligonucleotide sequences.
Hybridisation of the trans-DDP labelled nucleotide sequences to
their target results in stable interstrand cross-link of the
nucleotide sequences to the selected repetitive regions. These
nucleotide sequences may be modified to lack the 3' hydroxy
10   group thus disabling the nucleotide sequences to function as a
primer by the polymerase. Therefore amplification will be
blocked at the position of the cross-linked nucleotide
sequences and preferentially unblocked sequences, which do not
contain selected repetitive sequences will be amplified. As a
15   consequence, the amplification product can be used as a probe
for chromosome painting experiments directly without adding
repetitive nucleic acid(s) to the hybridisation mixture.
Alternatively, due to the reduced presence of repetitive
sequences significantly less repetitive nucleic acid(s) needs
20   to be added to the hybridisation mixture thereby significantly
improving the performance of a probe in the presence of
repetitive nucleic acid(s).
Blocking of amplification of specific sequences can also be
used in other PCR-applications or in vitro transcription
25   assays. Furthermore in all situations where an increased
stability of a connection between a DNA or a RNA-strand and its
target is required, selective cross-linking, for instance
through trans-DDP, can be applied. In the antisense technique,
translation of certain messenger RNAs (mRNA) is prevented by
30   the presence of selectively cross-linked antisense
oligonucleotides. Trans-DDP can create a stable connection
between these oligo's and the mRNA. In contrast, enhancing

translation by stabilising secondary structures of mRNAs is also possible with *trans*-DDP.

In one aspect of the invention is provided a process for the generation of a probe from which selected sequences, preferably repetitive sequences, are at least partially prevented from functioning as a probe (i.e. a nucleic acid provided with a label used for detection of the presence of said probe) through providing selected regions in a nucleic acid probe with interstrand cross-links. Said probe may be used in applications were nucleic acids probes are used for the detection of the presence of the probe such as but not limited to micro arrays, southern blots, northern blots, chromosome painting, etc. Advantages of a probe from which selected sequences are prevented from functioning of a probe are clear to the person skilled in the art and include but are not limited to improved specificity of said probe.

In one aspect of the invention is provided a process for the selected amplification of certain amplifiable sequences from a pool of amplifiable sequences comprising providing selected interstrand cross-links to decrease, or block at least in part, the amplification of a subset of amplifiable sequences, and subjecting said pool to an amplification reaction

In a preferred aspect of the invention a pool of amplifiable sequences is selected from sequences present in a chromosome. Preferably a pool of amplifiable sequences is selected from sequences of a part of a chromosome.

A collection of fragments produced during the selected amplification can be used as a probe for the detection of nucleic acid sequences. The probe may be labelled with conventional techniques or the probe may be labelled through the ULS, universal linkage system as described in (WO 92/01699,

WO 96/35696, WO 98/15564 and WO 98/45304). When the probe is made from sequences from an entire chromosome, the probe may be used to stain an entire chromosome. Similarly, when the probe is made from sequences from a part of a chromosome, the probe

5   may be used to stain a part of said chromosome.

Such labelled chromosomes or parts thereof may be used for the typing of a chromosome and/or a cell or for the identification of a disease.0000

10   One aspect of the invention provides a special labelling technique of bio-organic molecules, called COBRA (Combined Binary Ratio labelling). COBRA is based on the strategic combination of binary labelling and ratio labelling.

In a non limiting application the technique is used to achieve

15   FISH multiplicity of 24, 48, 96 or more based on existing technology and only requires a good digital fluorescence microscope.

In one aspect COBRA utilises combinatorial (i.e. binary) labelling and so-called ratio labelling for increasing the

20   number of identifiable colours for use in detection of nucleic acid in for instance cytogenetics. The COBRA labelling can be used for the labelling and/or detection of bio-organic molecules such as nucleic acid, protein, lipid and/or carbohydrate. A number of spectrally separated fluorophores is

25   used for ratio labelling, in such a way that two fluorophores are used to produce a certain colour. When this is applied for three fluorophores, and each pair of fluorophores results in 5 colours, a total of 12 colours is achieved (lower triangle in Figure 1). This primary probe set is directly fluorescently

30   labelled using methods such as nick translation, random primed labelling, PCR-labelling, and/or chemical labelling. A second set of 12 probes, recognising different targets is labelled exactly the same, but in addition is given a fluorophore. In

one example said fluorophore is a hapten, for instance biotin or digoxigenin. This hapten is developed using avidin or antibodies labelled with a fourth fluorescent label, spectrally well distinguishable from the three primary fluorophores used

5   for ratio labelling. Thus, the set of 12 is multiplied by 2, which results in 24 colours using 4 fluorophores only (two middle triangles in Figure 1), which is one fluorophore less than reported so far to accomplish staining of the 24 human chromosomes. Extra " free" fluorophores may be used to repeat

10   this process, exploring a second binary label, which again results in a doubling of the number of achievable colours (giving 48 colours) (upper triangles in Figure 1). Clearly, even stronger increments in number of colours are achievable if more than 12 primary colours are produced in the

15   basic triangle, either by using more than three fluorophores or by distinguishing more ratios. Mathematically, the total number of achievable COBRA colours can, at least in the case wherein two fluorophores are simultaneously used per target, be described as follows. Assume

20   that n fluorochromes are used for ratio labelling and assume that, as a non-limiting example, only 2 of those fluorochromes are simultaneously used per target, while additionally m fluorochromes can be binary labelled to the same target and r ratios can be resolved for ratio labelling, then the number of

25   different colours that can be distinguished is given by the following formula:

Formula I :
No. of colours = $(n +((r \times n!)/(2 \times (n - 2)!))) \times 2^{m}$

30

with : $2 \leq n \leq \infty,$

$0 \leq r \leq \infty$

$0 \leq m \leq \infty$

In one aspect, the invention provides a method for the generation of colours called COBRA, suitable for the labelling of probes, by mixing fluorochromes according to formula I, wherein n is the number of fluorochromes used for ratio labelling while, in this non-limiting example, only 2 of those fluorochromes are simultaneously used per target, m is the number of fluorochromes used to binary label the same target, and r is the number of ratios that can be resolved by ratio labelling.

The person skilled in the art will clearly be able to choose suitable fluorophores for use in COBRA.

The person skilled in the art will clearly be able to choose suitable ratios of fluorophores in ratio labelling.

In one embodiment of COBRA, the fluorochromes used for labelling may be selected from the group DEAC, Cy3®, fluorescein, Lissamine™ etc.

As used herein the term transition metal means a metal of group VIII of the periodic chart of the elements. A preferred transition metal for use in a cross-linking agent is platinum. In one aspect the invention provides a method for providing at least one selected sequence in a nucleic acid with interstrand cross-links comprising hybridising at least one selected single strand sequence with a complementary single strand nucleic acid wherein said selected sequence or said complementary nucleic acid or both comprise a cross-linking agent. In a preferred embodiment of the invention said selected interstrand cross-links hamper further hybridisation and/or replication of said selected sequences.

In another aspect the invention provides a method for the generation of a probe wherein at least one selected sequence in said probe is at least in part prevented from functioning as a

probe through providing said selected sequence with interstrand cross-links. Preferably said selected sequence comprises at least one repetitive sequence.

In one aspect of the invention is provided a method for the
5   selected amplification of certain amplifiable sequences from a pool of amplifiable sequences comprising producing a selected interstrand cross-linked nucleic acid or probe, wherein said selected interstrand cross-links are provided to decrease the amount of amplification of a subset of amplifiable sequences
10   and subjecting said pool to an amplification reaction.

Preferably a single stranded nucleic acid is prevented from taking part in said amplification through disabling the primer extension function of hybridised and cross-linked complementary single nucleic acid, preferably through modification of the 3'-
15   hydroxy group.

Preferably said pool of amplifiable sequences is selected from sequences present in a chromosome.

Following amplification said amplification will lead to a collection of amplified sequences, which among others may, upon
20   labelling, be used as a probe. When such pool of amplifiable sequences is selected from sequences present in a chromosome such a probe may be used in the preparation of a chromosome paint.

25   In a preferred embodiment of the invention a method referred to as COBRA is used for the labelling of a set of at least two bio-organic molecules with a set of at least two colours, comprising generating said set of colours through combining ratio labelling with binary labelling. In one embodiment of
30   COBRA, at least in the case wherein two fluorophores are simultaneously used per target, the total number of distinguishable colours of said combination can be calculated according to formula I,

No. of colours = $(n +((r \times n!)/(2 \times (n - 2)!))) \times 2^m$

wherein n is the number of fluorophores used for ratio labelling where in a non-limiting example, only 2 of those fluorochromes are simultaneously used per target, m is the number of fluorophores used to binary label the same target, and r is the number of ratios that can be resolved by ratio labelling.

with : $2 \leq n \leq \infty$,

$0 \leq r \leq \infty$

$0 \leq m \leq \infty$

In a preferred embodiment of COBRA, at least one of said bio-organic molecules comprises nucleic acid, protein, carbohydrate and/or lipid.

In another aspect of the invention is provided a method for simultaneous identification of sequences of at least one chromosome or part thereof, through the use of at least one probe, preferably prepared according to a method of the invention, wherein said probe is labelled according to a COBRA method for doubling the number of identifiable labels obtainable by ratio labelling, comprising adding to a first set of fluorophores, used for the ratio labelling of a first set of probes, a novel fluorophore and labelling a second set of probes.

In one embodiment of the invention a probe for the improved detection of chromosomes or parts thereof is provided.

In another embodiment, the invention provides the use of selected interstrand cross-links for decreasing the amount of amplified product of certain amplifiable sequences.

In another embodiment the invention provides the identification
of a disease through the typing of at least one chromosome
wherein at least one chromosome is labelled, with at least one
probe prepared according to the methods of the invention.

5   In yet another aspect of the invention a kit for the detection
of nucleic acid is provided, comprising at least one probe
obtainable by methods of the invention.
In yet another aspect the invention provides a kit for
performing the methods of the invention comprising at least one
10   probe labelled with a COBRA method.
In yet another aspect the invention provides a kit for
generating a probe according to the invention, comprising at
least a cross-linking agent, preferably linked to a single
stranded nucleic acid.
15   The invention further provides a kit for the detection of
nucleic acid comprising at least a collection of amplified
sequences or a probe. The invention further provides a kit for
performing the selective cross-linking of nucleic acid wherein
said kit comprising at least a cross-linking agent, preferably
20   linked to a single stranded nucleic acid.

The invention also provides a molecule comprising at least two
parts, cross-linked with a cross-linking agent, wherein said
cross-linking agent comprises a transition metal, preferably
25   platinum, wherein at least two of said parts comprise a
protein. Such a molecule is for instance produced with a method
wherein a protein is labelled with a ULS comprising a label,
wherein said label comprises a protein. Preferably said
molecule comprises at least two different proteins.
30

As used herein the term "interstrand cross-link" refers to a
physical link between a cross-linking agent and a double
stranded nucleic acid, wherein said physical link decreases the

propensity of a double stranded nucleic acid, to denaturate. Preferably but not necessarily an interstrand cross-link physically links the two complementary strands of the double stranded nucleic acid.

5 As used herein the term "physical link" is a covalent or non-covalent bond.

As used herein a probe is defined as collection of nucleic acid sequences comprising at least two different sequences, preferably labelled with a label facilitating detection of said

10 probe. Said probe may by used directly for the detection of for instance nucleic acid sequences or said probe may be manipulated according to the methods of the invention prior to the detection of for instance nucleic acid sequences.

As used herein the term "complementary" in relation to nucleic

15 acids is used functionally, meaning that the homology of a nucleic acid to a complementary nucleic acid is sufficiently high to allow hybridisation of a complementary nucleic acid to a nucleic acid under the desired stringent or non-stringent hybridisation conditions. This functional definition is

20 necessary to allow for different hybridisation conditions that may be utilised in practising the invention. One non-limiting example that illustrates the necessity for a functional definition is the cross-linking of a specific region which is repeated several times in a nucleic acid but where the repeated

25 regions vary slightly in the exact nucleotide sequence. Choosing a nucleotide sequence which is completely homologous to one region automatically implies that said nucleotide sequence is not completely homologous to the sequences of the repeated regions. The chosen sequence is however functionally

30 homologous to the sequences in the repeated regions , i.e. complementary, when the sequence dissimilarity of said repeated regions does not, under the chosen hybridisation conditions,

prevent hybridisation of the chosen sequence with said repeated regions.

Examples

Example 1

Blocking hybridisation of repetitive DNA by *trans*-DDP labelled

5   repetitive DNA.

Two slides with metaphase chromosomes are hybridised with Cy3-ULS labelled repetitive DNA. The first slide is prehybridised with unlabelled repetitive DNA. The second slide is prehybridised with *trans*-DDP labelled repetitive DNA.

10   Since hybridisation of *trans*-DDP labelled repetitive DNA to its target will create a stable interstrand connection, hybridisation of Cy3-ULS labelled DNA is prevented on the second slide. Therefore the Cy3 signal on the chromosomes is much lower on the second slide than on the first slide (see

15   table 1 for details).

Slides 1,2 and 3 are control slides. The numbers 1:0 represent the ratio amount repetitive DNA in relation to the amount trans-DDP. For the slides 2 and 3, no trans-DDP was used and there was no cross-linking. The acquired results thus have to

20   be seen as reference values. The results of slides 4 and 5 show that the repetitive DNA was over labelled with trans-DDP as a result of which the blocking of hybridisation was made more difficult (ratio 1:2). A non-saturated labelling of trans-DDP is depicted in slides 8 and 9. The best results were obtained

25   with a ratio of 1:1.

Example 2

Blocking amplification of specific sequences during PCR, by *trans*-DDP labelled dideoxy primer.

30   Two PCR reactions are run in parallel: In the first reaction PCR is performed on plasmid DNA with two sequence-specific primers (primer A and primer B). This reaction yields an amplification product of a defined length. In the second

reaction an identical PCR is performed. To the reaction mixture however, also two *trans*-DDP labelled oligonucleotides are added, which have a dideoxy nucleotide at their 3' end, and which are complementary to sequences within the region that can

5 be amplified by primer A and B. This second reaction will not yield a product, since amplification is blocked by the *trans*-DDP labelled oligonucleotides.

Example 3

10 Blocking repetitive sequences during DOP-PCR.

A DOP-PCR is performed in which amplification of repetitive sequences is blocked by adding *trans*-DDP labelled nucleotide sequences which lack the 3' hydroxy group and are complementary

15 to several repetitive sequences. With the amplification product an *in situ* hybridisation experiment on metaphase chromosomes is performed. In comparison to a non-blocked amplification product, this probe gives considerably less background on the non-target chromosomes.

20

Example 4

a. Two fluorochromes for ratio labelling (n=2), no ratios (r=0) and no binary label (m=0) results in 2 colours, as

25 expected.
b. Three fluorochromes for ratio labelling (n=3), 3 ratios (r=3) and 1 binary label (m=1) results in 24 colours (the situation that will be demonstrated in this paper)
c. Increasing the number of ratios to r=4 and the number of

30 fluorochromes for ratio labelling to 4 results in 28 colours.
d. Each binary fluorochrome results in doubling of the number of colours; that is to 56 (for 1) or to 112 (for 2).

The principle of this concept is demonstrated on 24 human
chromosomes using enzymatic labelling of probes and probe
mixing to accomplish ratio labelling (fluorescein, lissamine
and Cy5 as primary fluorophores and DEAC as combinatorial
5   label), as well as direct attachment of the colour code to the
probes using chemical labelling. In the latter DEAC, Cy3 and
Cy5 served as primary fluorophores, and Fluorescein or a
derivative was used as binary label.

10

Procedures

Multi-colour FISH staining of human chromosomes
Preparation of human metaphase chromosomes was performed as
15   described by Wiegant et al. Chromosomes from normal human
individuals as well as from *in vitro* cultured JVM-2 cells were
used. Probes for all chromosomes were obtained from Cytocell,
UK. All probe DNA was amplified by DOP-PCR to generate a set of
painting probes for all 24 human chromosomes.
20

Enzymatic labelling of probes

All probes were fluorescently labelled by incorporation of
labelled dUTPs either by PCR or nick translation using
25   fluorescein-, digoxigenin-dUTP (Boehringer Mannheim, Germany),
lissamine-dUTP (NEN life Science Products, USA) or Cy5-dUTP
(Amersham, UK). The digoxygenin-labelled probes were detected
indirectly using diethylaminocoumarin (DEAC, Molecular Probes,
USA).
30

Chemical labelling of probes using ULS (Universal Linkage System):

DEAC-ULS, Cy3-ULS and Cy5-ULS were chosen as primary
fluorophores and Fluorescein as combinatorial fourth label to
demonstrate digoxigenin-ULS (dig-ULS) labelled probes.
The following strategy was used to label and dissolve the ULS-
5   labelled probe set:

First, chromosome-specific painting probes for chromosomes
2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and X (100-400 ng) were
labelled in one reaction with dig-ULS according to the
manufacturers instructions. Thereafter, this probe set was
10   purified on a Qiagen quick spin column (Qiagen Inc., Valencia,
CA, USA) according to the manufacturers instructions. The
labelled probe mixture was eluted from the Qiagen column using
100 µl of 10 mM Tris.HCl pH 8.5.

Second, all chromosome-specific painting probes were
15   fluorescently labelled according to table 2 by mixing 30 µl of
the listed ULS compounds (or mixtures thereof) with 1 mg of
chromosome-specific painting probe DNA (all from Cytocell)
using DEAC-ULS (26.7 µM), Cy3-ULS (20 µM) and Cy5-ULS (13.3 µM)
in a final volume of 100 µl of water. In case probes were
20   labelled with mixtures of two different ULS-compounds, the ULS-
compounds were first mixed in the desired ratio before the
probe DNA was added. After 15 min incubation at 65°C, the
labelled probes were purified on Qiagen quick spin columns
(Qiagen Inc., Valencia, CA, USA). The labelled probes were
25   eluted from the Qiagen columns using 100 µl of 10 mM Tris.HCl
pH 8.5. Prior to the hybridisation, fluorescent ULS-labelled
probes where combined in amounts as indicated in the right
column of Table 2 together with the 100 µl of dig-ULS labelled
probe mixture from the first step. This probe mixture was then
30   ethanol precipitated in the presence of 10 x excess low
molecular weight fish sperm DNA (Boehringer Mannheim), and 3 x
excess human C_0t1-DNA (Gibco, BRL) (an alternative method for

suppression of repetitive sequences is presented below).
Thereafter the probe mixture was dissolved in 10 µl 50%
deionized formamide, 2xSSC, 50 mM sodium phosphate pH 7, 10 %
dextran sulfate. This 10 µl of probe mixture was used as
5   hybridisation solution.

FISH staining of human metaphase chromosomes:

    Slides with metaphase chromosomes were pre-treated with
RNaseA and pepsin according to Wiegant et al. The chromosome
10 preparations were denatured by incubating them 90 sec at 80°C
in 60% formamide, 2xSSC, pH 7 on a hot plate. After removal of
the coverslip the slides were dehydrated through an ethanol
series and air dried. Then, 10 µl hybridisation mixture was
applied under a 18x18 mm coverslip, sealed with rubber cement
15 and hybridisation was performed for 120 hrs at 37°C in a humid
chamber. The hybridisation mixture contained 50% formamide,
2xSSC, 50 mM sodium phosphate pH 7, 10 % dextran sulphate, 100-
500 ng of each DEAC-, Cy3- and Cy5-labeled probe (both single-
and ratio-labelled probes) (see Table 2), 100-400 ng of each
20 dig-ULS labelled probe, 3 x excess human C_0t1-DNA and 10 x
excess low molecular weight fish sperm DNA in 10 µl. Before
application, the probes were denatured for 10 min at 80°C,
followed by 60 min incubation at 37°C to allow pre-annealing
with the 3 times excess of C_0t1-DNA
25 After a 10 min post-hybridisation wash in 2xSSC/0.1% Tween 20
at 37°C to remove the coverslips, the slides were washed 2 x 5
min in 50 % formamide, 2XSSC, pH 7 at 44°C. This was followed
by 2 washes (5 min each) in 0.1xSSC at 60°C and a 5 min wash at
RT in TNT (0.1M Tris.HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20).
30 The DIG-ULS labelled probes were detected with a mouse
monoclonal antibody against digoxin (Sigma) followed by a
rabbit anti mouse antibody conjugated to FITC (Sigma).
Chromosomes were counterstained with DAPI. The slides were

embedded in Vectashield (when enzymatically labelled probes were used) or Citifluor (Agar, Stansted, UK) (when chemically labelled probes were used) prior to microscopical evaluation.

5   Digital imaging microscopy

Digital fluorescence imaging was performed using a Leica DM-RXA epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a 100-W mercury arc lamp and computer controlled filter wheels with excitation and emission filters for visualisation

10   of DEAC, Fluorescein, Cy3 and Cy5, using HQ-FITC, Pinkel set plus SP 570, HQ-Cy3, HQ-Cy5 and DEAC filter (Chroma Technology) respectively. DAPI was excited with UV light using block A. A 63x objective ((N.A. 1.32, PL APO, Leica) was used. Image acquisition and analysis was performed on a Cytovision

15   workstation (Applied Imaging, Sunderland, UK). This system consists of a PC (Pentium 133MHz processor, 24Mb Ram, 2.1 Gb disc and 17″ display) interfaced to a Coolview camera (Photonic Science). The camera has thermo-electric cooling, which allows on chip integration up to circa 30 seconds. Images

20   are digitised in an 8-bit 768 x 512 image format.

Image acquisition was performed as described before. Chromosomes were segmented interactively by thresholding the DAPI image. The segmented image was used as a mask for the colour image, which was composed of the 3 images corresponding

25   with the ratio labelled fluorochromes (green for DEAC, red for Cy3 and

blue for Cy5) and of the Fluorescein image. Note that this procedure does not require thresholding of the three colours. The fourth Fluorescein image was evaluated binary, that is

30   chromosomes with or without Fluorescein fluorescence were distinguished. This was performed by finding the optimal threshold in the histogram of the Fluorescein image for the pixels lying within the DAPI mask. Typically, two gaussion

distributions were observed, corresponding to Fluorescein positive and negative chromosomes .

Classification was performed in two steps: the chromosome classification was followed by a pixel classification to

5 detect eventual translocations. Chromosome classification was based mainly on the modal colour value of each chromosome, e.g. its position in one of the colour triangles (the one with or without the binary label), as shown in Figure 1. The shortest distance of the measured modal colour value of a chromosome to

10 the theoretical expected ratio colour of all chromosome classes was therefore calculated. In order to compensate for non-specific fluorescence contributions and to increase the robustness of the method the theoretical expected colour values were warped onto a triangle formed by the measured modal values

15 of the chromosomes with only one ratio colour. Besides the modal colour value also the length of the chromosomes was used for classification. Theoretically, the colour values of the chromosomes should correspond with the original probe ratios. In practice however, a more robust approach is obtained, when

20 a number of metaphases was used for training of the classifier. Following object classification, each pixel within a chromosome was classified on the basis of the shortest distance to the measured chromosome classes. The binary (fourth colour) information of each pixel was used to decide, within which

25 colour triangle

distance calculations should be performed. Assignment of classification colours is considered useful and foreseen, but was not implemented in the current software.

Finally, a karyogram was generated based on chromosome

30 classification showing the ratio colours, as described above. A karyogram, in which a pseudo colour was assigned to the corresponding chromosome class of each separate chromosome pixel was produced to facilitate the interactive detection of

chromosome translocations. When needed the DAPI banding image
was used for comparison purposes.

Results

5   A 24 colour COBRA staining procedure using four fluorophores
was applied to normal and abnormal chromosomes. The optimal
conditions for labelling of the probes and the final
composition of the probe set required some fine tuning, due to
the fact that some probes performed better than others.

10 Typically, less performing FISH probes were given such colour
combinations that colour overlap with other probes was
minimised.

Optimal staining results were obtained at prolonged
hybridisation times (5 days), although three days in many cases

15 was sufficient. The suppression of repetitive sequences was
found essential for selective staining of chromosomes.

Figure 2 shows how the 24 chromosomes occupy the colour space.
Typically, within a certain chromosome image, signal
intensities showed relatively large variations, due to local

20 differences in FISH intensity. The characteristic colour
however was sufficiently constant to form clusters, with a
defined angle within the three D colour space (Figure 2).
Although some chromosome clusters showed overlap, they were
well enough separated to be classified automatically using the

25 procedure described above.

Figure 3 shows the actual chromosome images and the resulting
karyogram. Integration times varied depending on the
fluorophore used and ranged from 0.5 to 20 sec. An
entire Cobra acquisition and analysis procedure typically

30 took approximately 1 min.

Applied to abnormal chromosomes as shown in the JVM cell line,
Cobra allowed for easy detection of abnormal chromosomes
(Figure 4). Essential in the ULS method is that in principle

each probe molecule contains the ratio code, making mixing obsolete. Ratio labelling of DEAC, Cy3 and Cy5 performed excellent, and could be well combined with binary fluorescein labelling. Results obtained with these probes are shown in

5   Figure 5.

The robustness of COBRA depended on the quality of the metaphase chromosomes obtained, as is the case for both automated analysis of Giemsa banded and FISH stained chromosomes. Good quality slides always resulted in images of

10   good signal to noise ratio that could be classified automatically, whereas user intervention increased with decreasing staining quality.

The Cobra principle combines the advantages of ratio labelling and binary labelling. It " settles" for making ratios of two

15   fluorophores only, but utilises the possibility of doubling the number of colours by introducing indirectly labelled haptens, that require a binary decision only. As shown, this approach is feasible and allows for identifying 24 human chromosomes using 4 fluorophores only.

20   The full potential of this approach has not been explored yet. So far only painting probes were used in Cobra. Considering the short exposure times, we anticipate that other type of probes such as YACs or PACs can be used in a similar approach.

25   As the mathematical equation shows, the number of colours particularly increases if more dyes or more ratios are used for the primary colour set. It has been shown that distinction of 6 or 7 ratio of two dyes is feasible.

Such an approach is best achievable if chemical labelling is

30   used. The ULS is advantageous for large scale production of quality controlled painting probes. In this context the COBRA strategy for efficient use of fluorophores can significantly

contribute to a further increase of MFISH multiplicity and thereby to further exploitation in cytogenetics.

Example 5

Prevention of cross-hybridisation between different HPV types.
Background: The KREATECH HPV typing probe 31, 33 gives on CaSki
5   cells a weak though clear hybridisation signal. CaSki cells are
HPV16. Can through the use of trans-DDP this undesired
hybridisation be prevented?
Scheme: select homologues sequences between HPV16 on the one
hand and HPV31 and 33 on the other hand. Label these sequences
10   with trans-DDP and irreversible cross-link these sequences
after hybridisation. The remaining sequences are HPV31 and/or
33 specific. After hybridisation with this DIG-ULS labelled
remaining fraction on CaSki cells no hybridisation is expected.

15   Example 6

Use of trans-DDP in filter hybridisations
Background: The prevention of hybridisation between sequences
that make the interpretation of the end result difficult. For
20   example repetitive sequences (for example in an intron) that
mask the signal of single copy. Or the suppression of generally
present sequences in a stage specific cDNA library favouring of
stage specific unique sequences (can be compared with
subtractive hybridisations).
25   Scheme: HPV16 is cloned in pSP64. After digestion with a
restriction enzyme that removes the insert from the plasmid,
both fragments are separated on agarose gel en blotted on a
filtermembrane. As probe the plasmid and the insert are
labelled with DIG-ULS. When this is used as such, two bands are
30   acquired after a hybridisation, i.e. the HPV16 en the pSP64
bands. However, through the addition of trans-DDP labelled
pSP64 DNA will the pSP64 sequences be irreversibly cross-linked
and even after denaturation they will not be capable anymore of

taking part in the subsequent hybridisation. As a result of this hybridisation only one predominant band is expected, i.e. the one specific for HPV16 (this in the ideal case). This example describes a cross-linking of homologues sequences in

5   solution. Reversal of the system and first performing a matrix hybridisation with trans-DDP pSP64 and subsequently after stripping of the filter a DIG-ULS pSP64/HPV16 hybridisation will result in a comparable result.

10   Example 7

In this example one aspect of the Cobra principle is implemented with TRANS-ULS labelled probes in an application using mFISH.

15   For the generation of a human chromosome 4 or chromosome 20 specific probe, a DOP-PCR is performed on human chromosome 4 or chromosome 20 preparations according to the procedure described in Multi-colour FISH staining of human chromosomes, in which amplification of repetitive sequences is blocked by adding

20   *trans*-DDP labelled nucleotide sequences which lack the 3' hydroxy group and are complementary to several repetitive sequences. The chromosome 4 specific probe was ratio labelled with DEAC-ULS and Cy3-ULS (50:50) according to the procedure described in Chemical labelling of probes using ULS. The

25   chromosome 20 specific probe was ratio labelled with DEAC-ULS and Cy3-ULS (50:50) and combinatorial labelled with Fluorescein according to the procedure described in Chemical labelling of probes using ULS.

Slides with metaphase chromosomes spreads of JVM-2 cells were

30   prepared and FISH- stained according to the procedure described in FISH staining of human metaphase chromosomes.

Results were visualised according to the procedure described under imaging microscopy.

When using the trans-ULS probes optimal staining results were obtained after surprisingly short hybridisation times, compared to the non-trans-ULS probes, in for instance example 4. Overnight hybridisation was often sufficient for staining.

5   Whereas for optimal results using non trans-ULS FISH-techniques as in example 4 hybridisation times of five days are optimal. The cobra labelling allowed clear and unambiguous typing of chromosome 4 and chromosome 20 in metaphase spreads of JVM-2 cells following overnight hybridisation with the probes.

10

Example 8: Blocking hybridisation of fluorophore labelled repetitive DNA by *trans*-DDP labelled repetitive DNA.

In this example we in essence repeated the experiments
15   described in example 1. A fluorescein-ULS labelled human chromosome 1 specific probe was hybridised *in situ* onto human metaphase chromosome spreads. For a person skilled in the art it is obvious that this type of probe contains non chromosome specific repetitive DNA sequences. Hybridisation of these
20   sequences was hindered often by adding excess of unlabelled human $C_o t$ 1 DNA. Here use is made of trans-DDP labelled repetitive DNA sequences to suppress hybridisation of non chromosome specific repetitive sequences present in a chromosome specific probe. All slides but one were denatured
25   and pre-incubated with trans-DDP labelled humane repetitive DNA. The ratio repetitive DNA : trans-DDP is given in table 1. Subsequently, all slides but one were denatured and fluorescein-ULS labelled probe was added to all slides. Hybridisation of *trans*-DDP labelled repetitive DNA to its
30   target created a stable interstrand connection, preventing hybridisation of fluorescein-ULS labelled DNA. Therefore, the intensity of the fluorescein signal on the chromosomes is reduced (see table 1 for details). Slides 1,2 and 3 are control

slides. For the slides 2 and 3, no trans-DDP was used and there was no cross-linking. Thus, the acquired results have to be seen as reference values. The results of slides 4 and 5 show that the repetitive DNA was over labelled with trans-DDP as a

5   result of which the blocking of hybridisation was made more difficult (ratio 1:2). A non-saturated labelling of trans-DDP is depicted in slides 8 and 9. The best results were obtained with a ratio of 1:1 (slide 6). For experimental details about ULS probe labelling and *in situ* hybridisation see example 4.

10

Example 9: Blocking amplification of specific sequences during PCR by *trans*-DDP labelled primers.

In this example we in essence repeated the experiments

15   described in example 2. Human Papillomavirus (HPV) type 16 primers HPVfor (5'-TCAAAAGCCACTGTGTCCTG-3') and HPVrev (5'-AACCACCCCCACTTCCAC-3') yielded a fragment of 945 bp in a polymerase chain reaction (PCR). Four internal primers were designed: primer TU16for1 (5'-AGAGCTGCAAAAAGGAGATTATTTGAAAGCGA-

20   3'), primer TU16for2 (5'-AGAGACAACTGATCTCTACTGTTATGAGCA-3'), primer TU16rev1 (5'-TCCTGTGCAGTAAACAACGCATGTGCTGTC-3'), and primer TU16rev2 (5'-CGTGTGTGCTTTGTACGCCACAACCGAAGCGTAGAGT-3'). These internal primers were pooled (0.125 µg/µl each). The primer mixture was labelled with 50 ng trans-ULS per µg primers

25   according to the standard ULS labelling protocol. Next, the oligonucleotide mix was column purified in order to remove free trans-ULS. Total genomic HPV 16 DNA (40 ng final) was mixed with trans-ULS labelled internal primers (120-160 ng final) in a solution of 6x SSC. This solution was denatured and incubated

30   at 60 °C for 1 hour. This step was repeated two more times and was followed by a column purification. Subsequent, a PCR amplification was carried out as follows: a PCR master mix consisting of a PCR buffer, HPVfor and HPVrev primers (10 µM

each, dNTPs (2.5 mM each), and Taq DNA polymerase (5 units) was added to a 0.5 ml PCR tube containing either (i) HPV 16 genomic DNA only, (ii) HPV 16 DNA and internal primers, or (iii) HPV 16 DNA cross-linked with trans-ULS labeled internal primers. The

5 PCR profile was: 95 °C for 2 minutes, 23 cycles of 95 °C for 45 seconds; 57 °C for 45 seconds; 72 °C for 1 minute, and 1 cycle of 95 °C for 45 seconds; 57 °C for 45 seconds; 72 °C for 15 minutes. Ten µl of each PCR amplified mix was run on a 1% agarose gel (see figure 6). Lane 1 shows the 945 bp fragment

10 (see above). The yield of the 945 bp fragment was reduced when the internal primers were added to the PCR mix (lane 2). When use was made of trans-ULS labeled internal primers no 945 bp PCR fragment was amplified. Irreversible cross-linking of the internal primers blocked the DNA polymerase chain elongation at

15 well defined positions within the 945 bp fragment. Similar results can be obtained when use is made of trans-ULS labelled dideoxy internal primers.

Example 10: Blocking repetitive sequences during DOP-PCR and

20 use of such probes in *in situ* hybridisation.

In this example we in essence repeated the experiments described in example 3. Human $C_o t$ 1 DNA was end labelled with ddATP according to the following protocol: 50 µg of $C_o t$ 1 DNA

25 was denatured at 90 °C for 10 minutes and mixed with TdT buffer, ddATP, and TdT (terminal transferase). The mixture was incubated at 37 °C over night and ethanol precipitated. Next, the 3'-ddATP human $C_o t$ 1 DNA was labelled with the cross-linking agent trans-ULS. Five µg of the DNA was mixed with

30 various amounts of trans-ULS, incubated at 85 °C for 30 minutes, and column purified. Best results were obtained with the 3'-ddATP human $C_o t$ 1 DNA:trans-ULS ratio of 1:0.3. The FISH

result of this sample is presented below. Human chromosome 1
painting probe was mixed with trans-ULS labelled 3'-ddATP human
C_0t 1 DNA (10 fold excess), denatured and allowed to hybridise
and interstrand cross-link at 65 °C overnight. Next, a small

5   aliquot of this sample was PCR amplified in two consecutive PCR
rounds, purified, and labelled with Cy3-ULS all according to
standard procedures. The chromosome 1 probe produced in this
way is deprived of high copy repetitive sequences (in this
particular example human C_0t 1 DNA homologous sequences). This

10   type of probe eliminates the use of human C_0t 1 DNA to suppress
cross-hybridisation of these repeats in *in situ* hybridisation
experiments. The applicability of the Cy3-ULS labelled repeat
free human chromosome 1 probe was demonstrated in FISH. The *in
situ* hybridisation was essentially the same as described in

15   example 4 and/or 12. The results are shown in Figure 7. A high
degree of non chromosome specific cross-hybridisation was seen
when use was made of the chromosome 1 probe not deprived of
high copy repetitive sequences without addition of human C_0t 1
DNA (Figure 7A). Addition of five fold excess of human C_0t 1

20   DNA largely suppressed non chromosome specific cross-
hybridisation. Human chromosome 1 could clearly be identified
(Figure 7B). Suppression of non chromosome specific cross-
hybridisation, without the need of large amounts of suppressor
DNA (in this case human C_0t 1 DNA), was obtained with probes as

25   described in this invention. Chromosome 1 could be clearly
identified when use was made of the trans-ULS treated repeat
deprived chromosome 1 specific probe (Figure 7C).

Example 11: Prevention of cross-hybridisation between different
30   Human Papillomavirus types.

In this example we in essence repeated the experiments
described in example 5. Several Human Papillomavirus (HPV)

types show a high degree of identity between their nucleotide
sequences. For example, HPV 18 and HPV 45 genomes show an
identity of 79.7%. The generation of a HPV 45 specific probe
free of HPV 18 homologous sequences can be made possible

5   through the use of a cross-linking agent. HPV 45 total genomic
DNA was labelled with DIG-ULS according to the standard ULS
labelling procedure. HPV 18 total genomic DNA was sonicated,
biotin end labelled, and labelled with trans-DDP at a
DNA:trans-ULS ratio of 1:1. The labelled DNAs were column

10   purified and mixed according to the following scheme:
Tube 1: DIG-ULS labelled HPV 45 + unlabelled HPV 18 (sonicated)
Tube 2: DIG-ULS labelled HPV 45 + biotinylated and trans-DDP
labelled HPV 18
The ratio HPV 45 : HPV 18 was 1:10 in both tubes. The DNAs,

15   dissolved in a solution of 6x SSC (final concentration), were
denatured and incubated at 65 °C for 5 hours. Streptavin coated
magnetic beads were added to these tubes and biotinylated DNAs
were removed from the solution. Consequently, tube 2 contains
mainly DIG-ULS labelled HPV45 DNA deprived from HPV 18

20   homologous sequences. The probes were denatured and mixed with
DIGEASYHYB solution at a final concentration of 25 ng/ml. HPV
45 and HPV 18 genomic DNA was spot blotted onto nylon membrane
strips at concentrations ranging from 1000 pg to 0.1 pg. Probe
mixes were added to the target strips and allowed to hybridise

25   over night at 42 °C. The result is shown in Figure 8. The DIG-
ULS labelled HPV 45 probe hybridises to itself irrespective of
the presence of unlabelled homologous HPV 18 DNA (lane 1). DIG-
ULS labelled HPV 45 unique sequences do hybridise to HPV 45
total genomic DNA but the signal is less strong due to a

30   reduced probe size (lane 2). DIG-ULS HPV 45 labelled unique
sequences do not hybridise to HPV 18 total genomic DNA under
the condition used in this experiment (lane 3).

Example 12: Use of trans-DDP in filter hybridisation

In this example we in essence repeated the experiments described in example 6. A cocktail of five oligonucleotides was labelled with 0.05 μg trans-ULS per μg DNA according to the standard ULS labelling procedure. Next, the trans-ULS labelled oligo cocktail was allowed to pre-hybridise with a cocktail of complementary Biotin-ULS labelled oligonucleotides (25 ng) at 65 °C over night. The trans-ULS labelled oligo cocktail was added at a five fold or ten fold excess, respectively. After being denatured these mixes were added to DIGEASYHYB buffer at a final concentration of 25 ng/ml of Biotin labelled complementary oligonucleotides. These mixes were allowed to hybridise at 37 °C for 6 hours to the cocktail of five oligonucleotides which were spotted onto nylon strips at various quantities ranging from 10000 to 1 pg. Hybridisation was visualised through the use of alkaline phosphatase labelled streptavidin and subsequent chemiluminescence detection. The results are shown in Figure 9. The following samples served as controls: (i) Biotin labelled complementary oligonucleotide cocktail not incubated with the trans-ULS labelled oligonucleotide cocktail (lane 1), and (ii) Biotin labelled complementary oligonucleotide cocktail pre-incubated with a 5 fold and 10 fold excess of the oligonucleotide cocktail (no trans-ULS) (lane 2 and 4, respectively). Lane 3 and 5 clearly show the effect of the cross-linking compound. The hybridisation signals are very weak and significantly less compared to the controls.

Example 13: Combined binary ratio labelling

In this example we in essence repeated the experiments
described in example 4.

5   a.   Two fluorochromes for ratio labelling (n=2), no ratios
(r=0) and no binary label (m=0) results in 2 colours, as
expected.

b.   Three fluorochromes for ratio labelling (n=3), 3 ratios
(r=3) and 1 binary label (m=1) results in 24 colours (the

10   situation that will be demonstrated in this example)

c.   Increasing the number of ratios to r=4 and the number of
fluorochromes for ratio labelling to 4 results in 28 colours.

d.   Each binary fluorochrome results in doubling of the
number of colours; that is to 56 (for 1) or to 112 (for 2).

15   The principle of this concept is demonstrated on 24 human
chromosomes using enzymatic labelling of probes and probe
mixing to accomplish ratio labelling (fluorescein, lissamine
and Cy5 as primary fluorophores and DEAC as combinatorial

20   label), as well as direct attachment of the colour code to the
probes using chemical labelling. In the latter DEAC, Cy3 and
Cy5 served as primary fluorophores, and Fluorescein or a
derivative was used as binary label.

25   Procedures:

I. Multi-colour FISH staining of human chromosomes
Preparation of human metaphase chromosomes was performed as
described by Wiegant et al (1993). Chromosomes from normal
human individuals as well as from *in vitro* cultured JVM-2 cells

30   were used. Probes for all chromosomes were obtained from
Cytocell, UK. All probe DNA was amplified by DOP-PCR to
generate a set of painting probes for all 24 human chromosomes.
II. Enzymatic labelling of probes

All probes were fluorescently labelled by incorporation of
labelled dUTPs either by PCR or nick translation using
fluorescein-, digoxigenin-dUTP (Boehringer Mannheim, Germany),
lissamine-dUTP (NEN Life Science Products, USA) or Cy5-dUTP

5   (Amersham, UK). The digoxygenin-labelled probes were detected
indirectly using diethylaminocoumarin (DEAC, Molecular Probes,
USA).

III. Chemical labelling of probes using ULS (Universal Linkage
System)

10   DEAC-ULS, Cy3-ULS and Cy5-ULS were chosen as primary
fluorophores and Fluorescein as combinatorial fourth label to
demonstrate digoxigenin-ULS (DIG-ULS) labelled probes.
The following strategy was used to label and dissolve the ULS-
labelled probe set:

15   First, chromosome-specific painting probes for
chromosomes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and X (100-
400 ng) were labelled in one reaction with DIG-ULS according to
the manufacturers instructions. Thereafter, this probe set was
purified on a Qiagen quick spin column (Qiagen Inc., Valencia,

20   CA, USA) according to the manufacturers instructions. The
labelled probe mixture was eluted from the Qiagen column using
100 μl of 10 mM Tris.HCl pH 8.5.
Second, all chromosome-specific painting probes were
fluorescently labelled according to table 2 by mixing 30 μl of

25   the listed ULS compounds (or mixtures thereof) with 1 mg of
chromosome-specific painting probe DNA using DEAC-ULS (26.7 μ
M), Cy3-ULS (20 μM) and Cy5-ULS (13.3 μM) in a final volume of
.100 μl of water. In case probes were labelled with mixtures of
two different ULS-compounds, the ULS-compounds were first mixed

30   in the desired ratio before the probe DNA was added. After 15
min incubation at 65°C, the labelled probes were purified on
Qiagen quick spin columns (Qiagen Inc., Valencia, CA, USA). The

labelled probes were eluted from the Qiagen columns using 100 μ
l of 10 mM Tris.HCl pH 8.5. Prior to the hybridisation,
fluorescent ULS-labelled probes where combined in amounts as
indicated in the right column of Table 2 together with the 100

5   μl of DIG-ULS labelled probe mixture from the first step. This
probe mixture was then ethanol precipitated in the presence of
10 x excess low molecular weight fish sperm DNA (Boehringer
Mannheim), and 3 x excess human C_0t 1 DNA (Gibco, BRL).
Thereafter the probe mixture was dissolved in 10 μl 50%

10 deionized formamide, 2xSSC, 50 mM sodium phosphate pH 7, 10 %
dextran sulfate. This 10 μl of probe mixture was used as
hybridisation solution.

IV. FISH staining of human metaphase chromosomes

Slides with metaphase chromosomes were pre-treated
15 with RNaseA and pepsin according to Wiegant et al (1991). The
chromosome preparations were denatured by incubating them 90
sec at 80°C in 60% formamide, 2xSSC, pH 7 on a hot plate. After
removal of the coverslip the slides were dehydrated through an
ethanol series and air dried. Then, 10 μl hybridisation mixture

20 was applied under a 18x18 mm coverslip, sealed with rubber
cement and hybridisation was performed for 120 hrs at 37°C in a
humid chamber. The hybridisation mixture contained 50%
formamide, 2xSSC, 50 mM sodium phosphate pH 7, 10 % dextran
sulphate, 100-500 ng of each DEAC-, Cy3- and Cy5-labeled probe

25 (both single- and ratio-labelled probes) (see Table 2), 100-400
ng of each DIG-ULS labelled probe, 3 x excess human C_0t 1 DNA
and 10 x excess low molecular weight fish sperm DNA in 10 μl.
Before application, the probes were denatured for 10 min at
80°C, followed by 60 min incubation at 37°C to allow pre-

30 annealing with the 3 times excess of C_0t 1 DNA.
After a 10 min post-hybridisation wash in 2xSSC/0.1% Tween 20
at 37°C to remove the coverslips, the slides were washed 2 x 5
min in 50 % formamide, 2XSSC, pH 7 at 44°C. This was followed

by 2 washes (5 min each) in 0.1xSSC at 60°C and a 5 min wash at RT in TNT (0.1M Tris.HCl pH 7.4, 0.15 M NaCl, 0.05% Tween 20). The DIG-ULS labelled probes were detected with a mouse monoclonal antibody against digoxin (Sigma) followed by a

5   rabbit anti mouse antibody conjugated to FITC (Sigma). Chromosomes were counterstained with DAPI. The slides were embedded in Vectashield® (when enzymatically labelled probes were used) or Citifluor® (Agar, Stansted, UK) (when chemically labelled probes were used) prior to microscopical evaluation.

10   V. Digital imaging microscopy
Digital fluorescence imaging was performed using a Leica DM-RXA epifluorescence microscope (Leica, Wetzlar, Germany) equipped with a 100-W mercury arc lamp and computer controlled filter wheels with excitation and emission filters for visualisation

15   of DEAC, Fluorescein, Cy3 and Cy5, using HQ-FITC, Pinkel set plus SP 570, HQ-Cy3, HQ-Cy5 and DEAC filter (Chroma Technology) respectively. DAPI was excited with UV light using block A. A 63x objective ((N.A. 1.32, PL APO, Leica) was used.
Image acquisition and analysis was performed on a Cytovision

20   workstation (Applied Imaging, Sunderland, UK). This system consists of a PC (Pentium 133MHz processor, 24Mb Ram, 2.1 Gb disc and 17" display) interfaced to a Coolview camera (Photonic Science). The camera has thermo-electric cooling, which allows on chip integration up to circa 30 seconds. Images

25   are digitised in an 8-bit 768 x 512 image format.
          Chromosomes were segmented interactively by thresholding the DAPI image. The segmented image was used as a mask for the colour image, which was composed of the 3 images corresponding with the ratio labelled fluorochromes (green for

30   DEAC, red for Cy3 and blue for Cy5) and of the Fluorescein image. Note that this procedure does not require thresholding of the three colours. The fourth Fluorescein image was evaluated binary, that is chromosomes with or without

Fluorescein fluorescence were distinguished. This was performed
by finding the optimal threshold in the histogram of the
Fluorescein image for the pixels lying within the DAPI mask.
Typically, two gaussion distributions were observed,

5   corresponding to Fluorescein positive and negative chromosomes
.

Classification was performed in two steps: the chromosome
classification was followed by a pixel classification to detect
eventual translocations. Chromosome classification was based

10   mainly on the modal colour value of each chromosome, e.g. its
position in one of the colour triangles (the one with or
without the binary label), as shown in Figure 1. The shortest
distance of the measured modal colour value of a chromosome to
the theoretical expected ratio colour of all chromosome classes

15   was therefore calculated. In order to compensate for non-
specific fluorescence contributions and to increase the
robustness of the method the theoretical expected colour values
were warped onto a triangle formed by the measured modal values
of the chromosomes with only one ratio colour. Besides the

20   modal colour value also the length of the chromosomes was used
for classification. Theoretically, the colour values of the
chromosomes should correspond with the original probe ratios.
In practice however, a more robust approach is obtained, when a
number of metaphases was used for training of the classifier.

25   Following object classification, each pixel within a chromosome
was classified on the basis of the shortest distance to the
measured chromosome classes. The binary (fourth colour)
information of each pixel was used to decide, within which
colour triangle distance calculations should be performed.

30   Assignment of classification colours is considered useful and
foreseen, but was not implemented in the current software.
Finally, a karyogram was generated based on chromosome
classification showing the ratio colours, as described above. A

karyogram, in which a pseudo colour was assigned to the corresponding chromosome class of each separate chromosome pixel was produced to facilitate the interactive detection of chromosome translocations. When needed the DAPI banding image

5   was used for comparison purposes.

Results:

A 24 colour COBRA staining procedure using four fluorophores was applied to normal and abnormal chromosomes. The optimal

10 conditions for labelling of the probes and the final composition of the probe set required some fine tuning, due to the fact that some probes performed better than others. Typically, less performing FISH probes were given such colour combinations that colour overlap with other probes was

15 minimised.

Optimal staining results were obtained at prolonged hybridisation times (5 days), although three days in many cases was sufficient. The suppression of repetitive sequences was found essential for selective staining of chromosomes.

20 Figure 2 shows how the 24 chromosomes occupy the colour space. Typically, within a certain chromosome image, signal intensities showed relatively large variations, due to local differences in FISH intensity. The characteristic colour however was sufficiently constant to form clusters, with a

25 defined angle within the three D colour space (Figure 2). Although some chromosome clusters showed overlap, they were well enough separated to be classified automatically using the procedure described above.

Figure 3 shows the actual chromosome images and the resulting

30 karyogram. Integration times varied depending on the fluorophore used and ranged from 0.5 to 20 sec. An entire COBRA acquisition and analysis procedure typically took approximately 1 min.

Applied to abnormal chromosomes as shown in the JVM cell line, COBRA allowed for easy detection of abnormal chromosomes (Figure 4). Essential in the ULS method is that in principle each probe molecule contains the ratio code, making mixing

5   obsolete. Ratio labelling of DEAC, Cy3 and Cy5 performed excellent, and could be well combined with binary fluorescein labelling. Results obtained with these probes are shown in Figure 5.

The robustness of COBRA depended on the quality of the

10   metaphase chromosomes obtained, as is the case for both automated analysis of Giemsa banded and FISH stained chromosomes. Good quality slides always resulted in images of good signal to noise ratio that could be classified automatically, whereas user intervention increased with

15   decreasing staining quality.

The COBRA principle combines the advantages of ratio labelling and binary labelling. It "settles" for making ratios of two fluorophores only, but utilises the possibility of doubling the number of colours by introducing indirectly labelled haptens,

20   that require a binary decision only. As shown, this approach is feasible and allows for identifying 24 human chromosomes using 4 fluorophores only.

The full potential of this approach has not been explored yet. So far only painting probes were used in COBRA.

25   Considering the short exposure times, we anticipate that other type of probes such as YACs or PACs can be used in a similar approach.

As the mathematical equation shows, the number of colours particularly increases if more dyes or more ratios are used for

30   the primary colour set. It has been shown that distinction of 6 or 7 ratio of two dyes is feasible.

Such an approach is best achievable if chemical labelling is used. The ULS is advantageous for large scale production of

quality controlled painting probes. In this context the COBRA strategy for efficient use of fluorophores can significantly contribute to a further increase of MFISH multiplicity and thereby to further exploitation in cytogenetics.

5

Example 14: COBRA with repeat free whole chromosome probes

In this example one aspect of the COBRA principle is implemented with trans-ULS labelled probes in a mFISH
10 application. In this example we in essence repeated the experiments described in example 7.

For the generation of a human chromosome 1 and chromosome 8 specific probe deprived of repetitive sequences a DOP-PCR was performed on human chromosome 1 and chromosome 8 preparations
15 according to the procedure described in example 2. Amplification of repetitive sequences was blocked by *trans*-DDP labelled complementary repetitive nucleotide sequences which lacked the 3' hydroxy group. Labelling of probes was as described in chemical labelling of probes using ULS in example
20 4. The chromosome 8 specific probe was ratio labelled with Cy3-ULS and Cy5-ULS (50:50). The chromosome 1 specific probe was ratio labelled with Cy3-ULS and Cy5-ULS (50:50) and combinatorial labelled with dGREEN-ULS.

Slides with metaphase chromosomes spreads were prepared and
25 FISH-stained according to the procedure described in FISH staining of human metaphase chromosomes in example 4.

Results are depicted in Figure 10.

When using the trans-ULS probes optimal staining results were obtained after surprisingly short hybridisation times, compared
30 to the non-trans-ULS probes, in for instance example 4. Overnight hybridisation was often sufficient for staining whereas for non trans-ULS FISH-techniques, as in example 4, hybridisation times of several days are optimal. The COBRA

labelling allowed clear and unambiguous typing of chromosome 1 and chromosome 8 in metaphase spreads following overnight hybridisation with the probes.

5   Example 15: COBRA based labelling of proteins

In resemblance with COBRA labelling and detection of nucleic acids the COBRA method offers the possibility to detect many proteins simultaneously, even when the number of labels

10   available is limited (less then the number of proteins to be investigated). Important is the broad applicability of the ULS labelling technology in labelling bio-organic molecules. This example demonstrates both the successful use of ULS labels in labelling proteins and shows proof of principle of COBRA

15   labelling and detection of proteins.

The proteins that are going to be detected in this example are avidin and bovine serum albumin (BSA). These two proteins were single and multiple labelled with ULS

20   labels in an aqueous solution at physiological conditions.

The labels with which the proteins were labelled are Flu-ULS (fluorescein), DNP-ULS (dinitrophenol), and DIG-ULS (digoxigenin). The labels used for ratio labelling were Flu and

25   DNP. Digoxigenin was the binary label. The generation of the various labelling solutions was performed as follows. For single labelling of avidin or BSA, which served as a control for the labelling of proteins with ULS per se, the

30   label consisted of 1 mg/ml of either Flu-ULS, DNP-ULS or DIG-ULS. For ratio labelling the label consisted of Flu-ULS and DNP-ULS, mixed in a 1:1 ratio.

For the combined ratio-binary labelling, the labelling mixture consisted of the ratio labels + the binary label (DIG-ULS) in an equimolar amount.

5 The proteins were incubated with the labels according to the description below.

Each protein (2μg/μl in 0.5 PBS) was labelled by mixing 50 μl of the protein solution with 50 μl of a stock solution of 1 mg/ml label in 0.5 x PBS of

10   - the single label either Flu-ULS, DNP-ULS or DIG-ULS
      - the ratio-label (Flu-ULS:DNP-ULS)
      - the ratio-binary label (Flu-ULS:DNP-ULS + DIG-ULS)

Labelling was performed at 37 °C for 1 hour. The ULS labelled proteins were spotted on nitrocellulose membranes. The solutions containing the labelled protein were spotted on several strips (1 ul per spot). Next the spots were air dried and subsequently the filters were blocked for 15 min. in blocking solution (1 x Blocking medium of Boehringer Mannheim, cat no. 1 585 762) in the maleic buffer according to the manufactures instructions. Next, the filters were incubated in a solution comprising the same blocking solution, 1 mg/ml BSA, and alkaline phosphatase (AP) labelled antibodies. The antibodies used are alkaline phosphatase labelled antibodies specific for digoxigenin (sheep anti-digoxigenin-AP; 1:5000; Roche Molecular Biochemicals 1 093 274), dinitrophenol (rabbit anti-DNP-AP; 1:1000; Sigma D5103), and fluorescein (sheep anti-fluorescein-AP; 1:5000; Roche Molecular Biochemicals 1 426 338). This incubation took place at room temperature for 30 min. Next, the filters were washed 3 times for 5 min. in TNT buffer followed by 2 washes in water for 2 min. each. The NBT/BCIP detection system for AP was applied according to the manufactures instructions. The AP reaction was allowed to take

place for 16 hours in the dark at room temperature. The
reaction was stopped by washing the filters in 1 x TE buffer
for 15 min.

The results are shown in Table 3.

5   Column Av-Flu combined with rows 1 - 4 of table 3 depicts the
results of avidin labelled with Flu-ULS and detected with AP
labelled antibodies. Only those spots showed a clear positive
signal where AP anti-Flu was present. This demonstrates that
avidin was successfully labelled with Flu-ULS.

10   Taken together, and in a similar fashion, Table 3 shows that
both avidin and BSA could be labelled with the different labels
as described above. Also, the proteins could be labelled with
various ULS labels simultaneously. In this particular example
the two proteins could not be distinguished from each other

15   based on their ratio labels only (Table 3, row 3 and 7).
Labelling one protein in a binary fashion made possible to
distinguish the two proteins (Table 3, row 4 and 9).
Table 3 shows that both proteins can be labelled with different
ULS labels and COBRA labelling could be successfully applied to

20   proteins.

Alternatively, <u>simultaneous</u> detection of proteins labelled
according to the COBRA principle can be made possible through
the use of label specific detection systems. Note that the

25   principle of COBRA labelling is independent of the type of
target molecules.

Brief description of the drawings

Figure 1: Principle of COBRA. The primary set of 12 ratio colours is doubled each time an independent binary label is introduced, resulting in 24 colours for 1 hapten, and in 48 colours for 2 haptens.

Figure 2:
Human chromosomes were stained in 24 colours using the COBRA principle.
For each of the 24 chromosomes the fluorescence intensity was plotted in a three dimensional colour space. Each coloured dot represents the measured colour intensity of an image point (pixel) of a certain chromosome.
(a): three primary colours (fluorescein, lissamine, Cy 5); without binary DEAC label;
(b): idem, with binary DEAC label.
    Note: Figure 1 is a schematic top view of the 2 x 12 clusters seen at equal x,y,z values of the measured data shown in this figure.

Figure 3:
Normal human chromosomes labelled by COBRA in 24 colours (same data as Figure 2).
(a) Image (12 colours) resulting from the three primary dyes used in ratio labelling;
(b) DEAC image of the same metaphase cell;
(c) Karyogram resulting from the combination of image (a) and (b) and automated classification

Figure 4:
COBRA (24 colours) applied to a JVM cell line (B-prolymphocytic leukemia) showing translocations t(11,14), t(3,8) and t(1,15).

Figure 5:

Results of ratio labelling obtained using chemical labelling (ULS system). The fluorophores DEAC, Cy3 and Cy5 were used as primary labels for ratio labelling. The DIG-ULS labelled second set of 12 probes was demonstrated indirectly using fluorescein labelled immunoconjugates.

(a) Image (12 colours) resulting from the three primary dyes used for ratio labelling;

(b) Image of the binary (fourth) label (fluorescein, but shown in blue false colour);

(c) Thresholded image (b) to indentify the fluorescein positive and negative sets of chromosomes; Note: DEAC is used as direct probe label here (through ULS), whereas in figure 2,3 and 4 is was used as binary label (as immunoconjugate).

Figure 6: Blocking of PCR with internal trans-ULS labelled oligonucleotides. PCR of a DNA fragment with a forward and reverse primer (lane 1) was inhibited by pre-hybridisation of the target DNA with a pool of 4 internal oligonucleotides (lane 2) and completely blocked if the internal oligonucleotides were labelled with trans-ULS (lane 3).

Figure 7: FISH with repeat free human chromosome 1 specific probe. Figure 7A shows FISH image of a human metaphase spread probe with human chromosome 1 probe. No use was made of human $C_o t$ 1 DNA. Note the high degree of non specific staining of other chromosomes present in the complement of the human genome which makes identification of chromosome 1 difficult. Reduced non specific cross-hybridisation of repetitive sequences present in the chromosome 1 specific probe was obtained by preannealing the probe with a 5 fold excess of human $C_o t$ 1 DNA (1 hour at 37 °C). Chromosome 1 could be identified with ease

(Figure 7B). The Chromosome 1 specific painting probe deprived from highly repetitive sequences through the use of trans-ULS allowed unambiguous identification of chromosome 1, without the use of human $C_o t$ 1 DNA, among the other chromosomes present in

5   the complement of the human genome (Figure 7C).

Figure 8: Filter hybridisation analysis of HPV18 deprived homologous sequences in a HPV45 probe using the trans-ULS technology. Biotin end-labelled and trans-ULS labelled

10   sonicated HPV18 DNA is pre-associated with DIG-ULS labelled HPV45 DNA. Biotin labelled HPV18-HPV45 hybrids are subsequently removed from the probe mixture using streptavidin magnetic beads. Detection of hybridisation is done using αDIG-AP antibodies in combination with CDP-Star™.

15   Lane 1, hybridisation of a DIG-ULS labelled HPV45 probe, pre-associated with non-labelled sonicated HPV18 DNA on spotted genomic HPV45;

Lane 2, hybridisation of a DIG-ULS labelled HPV45 probe, pre-associated with Biotin end-labelled and trans-ULS labelled

20   HPV18 on spotted genomic HPV45;

Lane 3, hybridisation of a DIG-ULS labelled HPV45 probe, pre-associated with Biotin end-labelled and trans-ULS labelled HPV18, on spotted genomic HPV18.

25   Figure 9: Filter hybridisation analysis of an oligonucleotide mixture probes with biotinylated complementary oligonucleotides.

Lane 1, Biotin labelled complementary oligonucleotide mix hybridised on spotted oligonucleotides;

30   Lane 2, Biotin labelled complementary oligonucleotide mix pre-associated with a five fold excess of the oligonucleotides and hybridised on spotted oligonucleotides;

Lane 3, Biotin labelled complementary oligonucleotide mix pre-associated with a five fold excess of trans-ULS labelled oligonucleotides and hybridised on spotted oligonucleotides;

Lane 4, Biotin labelled complementary oligonucleotide mix pre-associated with a ten fold excess of the oligonucleotides and hybridised on spotted oligonucleotides;

Lane 5, Biotin labelled complementary oligonucleotide mix pre-associated with a ten fold excess of trans-ULS labelled oligonucleotides and hybridised on spotted oligonucleotides.

Figure 10: COBRA with repeat free whole chromosome probes. Human chromosome paints specific for chromosome 1 and 8, depleted from repetitive sequences and COBRA labelled according to the invention, were probe onto human metaphase chromosome spreads. No use was made of human $C_o t$ 1 DNA. Chromosome 1 and 8 were ratio labelled with Cy3-ULS and Cy5-ULS whereas chromosome 1 was binary labelled only (dGREEN-ULS). Although the two chromosomes have the same ratio labels (Cy3 and Cy5) and ratio (50:50), the binary label made possible to discriminate the two chromosomes from each other.

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Table 1 repetitive DNA test with trans-DDP ULS

slide	Denat. 1	repetitive DNA:ULS		Denat.(2)	Probe	FISH result
1	no	no		yes	c#1-Flu	++++
2	yes	yes	1:0	yes	c#1-Flu	++
3	yes	yes	1:0	no	c#1-Flu	-
4	yes	yes	1:2	no	c#1-Flu	++
5	yes	yes	1:2	yes	c#1-Flu	++
6	yes	yes	1:1	yes	c#1-Flu	+/-
7	yes	yes	2:1	yes	c#1-Flu	+
8	yes	yes	4:1	yes	c#1-Flu	++
9	yes	yes	8:1	yes	c#1-Flu	++

Table 2

Chrom No.	μl DEAC ULS (26.7 μM)	μl Cy3 ULS (20 μM)	μl Cy5 ULS (13.3 μM)	ng probe DNA in hybrid. Mix
1	30	0	0	500
2	0	0	30	300
3	0	30	0	400
4	30	0	0	500
5	0	0	30	600
6	0	30	0	500
7	22.5	9	0	300
8	22.5	0	9	400
9	0	22.5	7.5	500
10	22.5	9	0	500
11	7.5	0	22.5	500
12	0	22.5	7.5	400
13	22.5	0	9	300
14	19.5	0	15	300
15	0	18	15	300
16	15	18	0	300
17	19.5	0	15	300
18	0	18	15	400
19	7.5	22.5	0	400
20	7.5	0	22.5	400
21	0	10.5	22.5	300
22	7.5	22.5	0	400
X	0	10.5	22.5	400
Y	15	18	0	100

Table 3

Row	Antibody	Av-Flu	Av-DNP	Av-Flu/DNP		
1	AP anti-Flu	+	−	+		
2	AP anti-DNP	−	+	+		
3	AP anti-Flu AP anti-DNP	+	+	+		
4	AP anti-DIG	−	−	−		
		BSA-Flu	BSA-DNP	BSA-Flu/DNP	BSA-DIG	BSA-Flu/DNP/DIG
5	AP anti-Flu	+	−	+	−	+
6	AP anti-DNP	−	+	+	−	+
7	AP anti-Flu AP anti-DNP	+	+	+	−	+
8	AP anti-DIG	−	−	−	+	+
9	AP anti-Flu AP anti-DNP AP anti-DIG	+	+	+	+	+